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13. ABSTRACT ***(Maximum 200 Words)***

We have established adenovirus transduction as a method for the short term alteration of gene expression in the mammary epithelium. Detailed conditions for transducing up to 30% of the cells in the mammary gland of the late pregnant mouse have been worked out, transduction with adenovirus and expression of green fluorescent protein, GFP, have been shown not to interfere with function of the mammary cells in which they are expressed. The transgene is maintained for seven days in the lactating mouse after transduction in late pregnancy without inflammation and without interfering with the function of the gland. Within this time frame function altering transgenes can be studied. However, longer term transduction is associated with significant inflammation. Transgenic technology has been used to evaluate the effects of an activated prolactin receptor, aPRL, and an activated member of the prolactin signal transduction pathway, Akt on the mammary epithelium. The latter has been shown to prolong mammary involution and preliminary results suggest it may enhance tumorigenesis. Adenovirus transduction is appropriate for short term experiments, up to 7 days, but cannot be used for long term expression of foreign proteins because of inflammatory reactions.

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INTRODUCTION

The propose research sought to:

1. Explore the use of adenovirus vectors for localized transduction of the mammary epithelium *in vivo* with genes of known tumorigenic potential.
2. Utilize a constitutively active prolactin receptor to test the hypothesis that long term stimulation of the prolactin receptor pathway promotes mammary tumorigenesis.
3. Use antibodies specific for signal transduction intermediates to investigate the state of signaling molecules in specific cells.

BODY

Task 1: Construction of Adenovirus vectors.

Adenovirus vectors were constructed with lacZ and green fluorescent protein (GFP) cDNAs. In addition we have constructed an adenovirus vector with the cytoplasmic tail of the tight junction protein occludin. This construct was not included in the original statement of work but was constructed for the purpose stated in task 2.

Task 2: Studies of efficacy and persistence of adenovirus vectors in various reproductive stages.

A. This task has extended through the entire three years of the project and has constituted the major portion of the work. We have injected a virus with the LacZ gene and the results are shown in the section in Figure 1 where about 20% of the cells have been transduced. We then went on to studies using a virus encoding the GFP protein. Again we examined

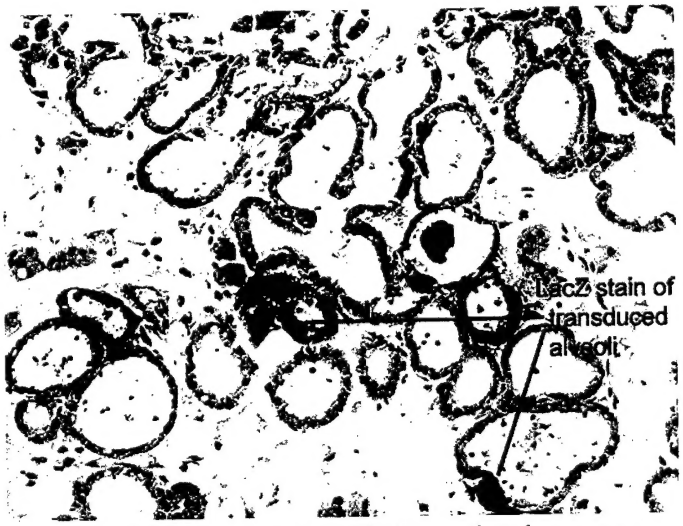

Figure 1. Histological section of lacZ transduced mammary gland. Note stained cells in extended interstitial space.

a. The histology of the gland
b. The extent of transduction.
c. The state of the tight junctions using a test derived in this l laboratory, up-the teat injection of ^{14}C-sucrose and monitoring its appearance in the blood stream. Under normal conditions in these mice, there should be no appearance of the tracer in the blood, because the tight junctions between epithelial cells are tightly closed.

Our findings:

a. With concentrations of virus of 10^8 pfu and above a severe inflammatory response could be observed histologically, the transduction efficiency was not markedly improved using the LacZ marker, and the tight junctions were open.

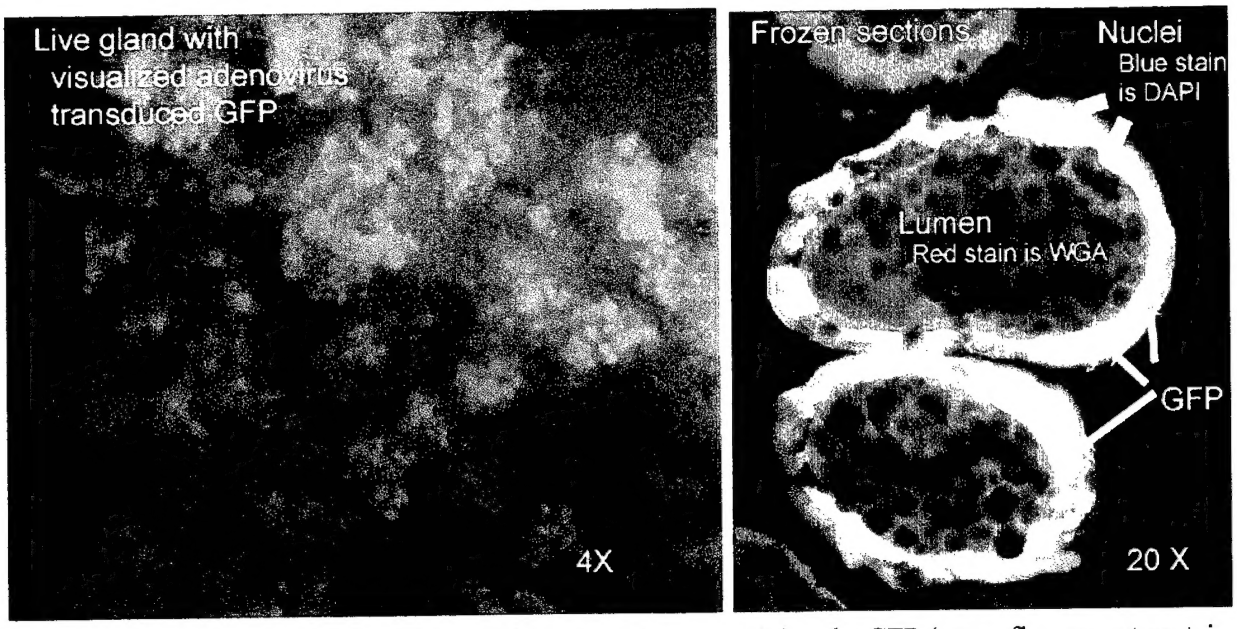

Figure 2. Images from Gland transduced with Adenovirus containing the GFP (green fluorescent protein gene) at late pregnancy and evaluated during lactation. Left: Gland visualized in the live animal under the dissecting microscope. Sections of gland stained with Cy3-labelel wheat germ agglutinin for apical mucins (WGA) and DAPI for nuclei.

b. With concentrations of virus of 10^7 using lacZ the junctions were closed but the gland showed patchy transduction and some signs of inflammation.

c. With concentrations of virus of 10^7 pfu using GFP as a marker and diluting the virus to the highest volume that could be injected into the 4th mammary gland (about 200 µl) at late pregnancy, we obtained no inflammation and highly efficient transduction when animals were sacrificed 3 to 7 days after transduction (Figure 2).

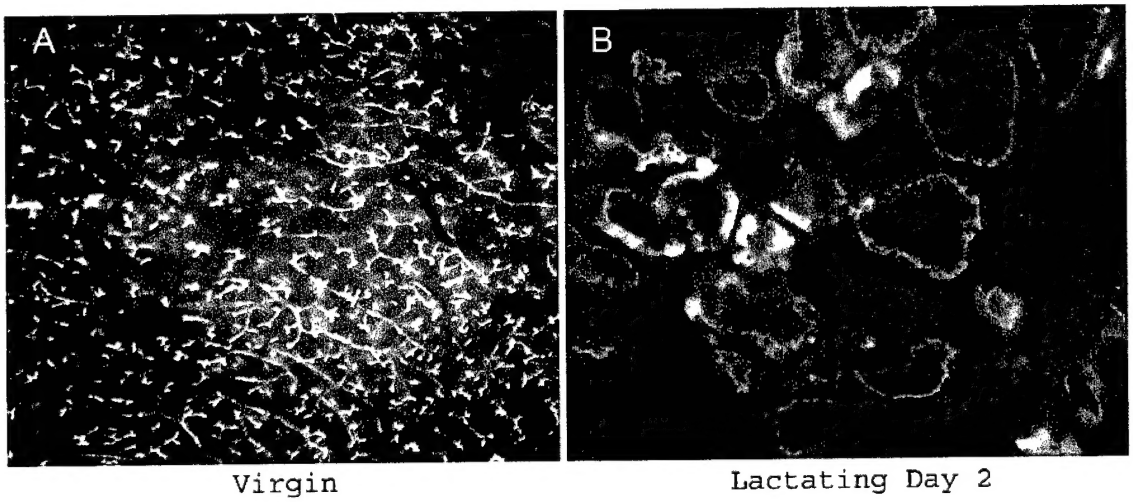

Figure 3. A.. Transduction in the virgin gland. Ductal cells appear to be transduced throughout their lengths. B. Transduction in the lactating gland. Gland injected at pregnancy day 17 and mouse sacrificed at pregnancy day 2. Most of the alveoli have transduced cells with various degrees of brightness.

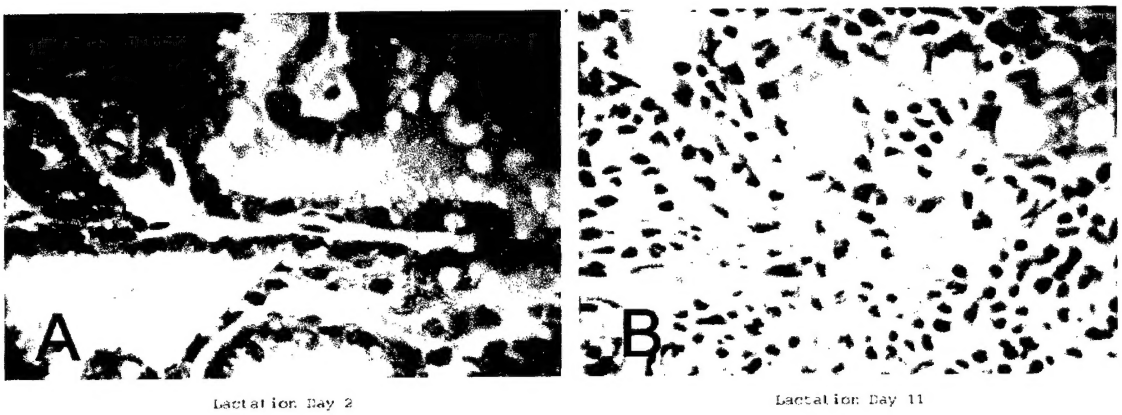

Figure 4. Histological sections of lactating mammary gland after adenoviral transduction on day 17 of pregnancy. A. Day 2 lactating gland. Milk secretion is normal and no signs of lymphocyte infiltration are seen. B. Transduced lactating gland on day 11 of lactation, 13 days after transduction. The gland is disorganized and seriously infiltrated with lymphocytes.

B. Effect of reproductive stage on adenoviral transduction.

We found that efficient transduction of alveoli occurs with injection into virgin animals (Figure 3A) and at late pregnancy (Figure 3B) but not in early pregnancy or lactation. In early pregnancy the cells are dividing rapidly, probably resulting in dilution and/or loss of adenovirus. There are two possible causes for the limited transduction in lactation: either milk products interfere with viral binding to the epithelium or viral receptors are not expressed on that apical surfaces of the epithelium.. We focused our efforts on mice transduced on day 18 of pregnancy. These mice were examined for extent of transduction, inflammation and other functional parameters on days 2, 5, 10 and 15 days of lactation. Table 1 shows our current results at different reproductive stages.

Table 1.

Stage at Transduction	Stage at Sacrifice	Degree of Transduction	Tight junction permeability	Inflammatory cells
Virgin	3 days after transduction	High (data under evaluation)	Moderate	High
3 day pregnant	6 day pregnant	None	Not done	None
17 day pregnant	Day 2 Lactating	10 - 30% of cells	Low	None
17 day pregnant	Day 5 Lactating	10 - 30% of cells	Low	Few
17 day pregnant	Day 10 Lactating	10 - 30% of cells	Moderate	Present
17 day pregnant	Day 15 Lactating	10 - 30% of cells	Moderate	High
Lactating day 4	Lactating day 7	Very low	Low	Present

As the table shows, we achieve reasonably high transduction levels. No signs of inflammation were seen up to day 5 of lactation (figure 4A) but at days 10 or 11 and 15, tight junction permeability, assessed by injection of the 14C-sucrose up the teat, was high and significant numbers of inflammatory cells were observed infiltrating the epithelium (Figure 4B).

C. Effect of optimal adenoviral transduction on physiological parameters

On the basis of the results in part B, we have determined that adenoviral transduction in late pregnancy is the most efficient and does not result in inflammation of the gland. Using this protocol in B we examined tight junction closure using the ^{14}C-sucrose protocol and milk secretion by morphological assessment of after viral transduction. In these animals there was no passage of ^{14}C-sucrose into the blood stream, indicating that the junctions close normally during the transition from pregnancy to lactation. In addition milk fat droplets appeared to be secreted normally judging from the immunocytochemical localization of the milk fat globule protein, xanthine oxidase and the presence of casein in both cells and alveolar lumina. Therefore transduction with virus coding for GFP does not alter cell function. Normal lactating transduced glands are shown in Figures 2 and 4 A.

D. Can adenoviral transduction of a dominant negative gene alter mammary function?

Because it appeared from studies in transgenic mice that a constitutively active prolactin receptor had little or no effect on mammary tumorigenesis (see below) and therefore would not serve as a proof of principle of function, we decided that a different *proof of principle* was essential at this junction. In essence it is necessary to show that adenoviral transduction can alter physiological function *in vivo.* Because we are very familiar with the test for tight junction integrity described above and because a change in tight junction permeability is an accompaniment of the transition from pregnancy to lactation, we made an adenoviral construct containing the cytoplasmic tail of the tight junction protein occludin. Using a retroviral system this gene product has previously been shown to interfere with tight junction closure in tissue culture systems (1). We hypothesized that this gene would interfere with tight junction closure in the *in vivo* system. We found that transduction of this gene not only disrupted tight junctions it actually caused programmed cell death in a very stereotypic manner. In particular we observed the following sequency of events:

a. Transduced occludin localized to the plasma membrane within 24 hours
b. Transduced occludin began to vesiculate at 48 hours with other tight junction components also appearing in the vesicles.
c. Nuclei condensation began soon there after and the nuclei were ejected from the cells.
d. Cytoplasmic blebs containing GFP (the marker for transduction) and the tight junction bundling protein, fascin, were left after the nucleus disappeared.

These results were similar to results observed with the same construct in cultured cells providing a proof of principle that transduction of specific genes can elicit specific and, in this case, predicted results.

Task 3. Transduction with known oncogenes

Because the time over which meaningful transduction can be achieved is very short, this experiment was not attempted. Potentially retrovirus with oncogenes could be injected in early pregnancy when it would integrate into the cells and provide long term expression. However, this experiment is beyond the scope of the proposed experiments.

Task 4. Transduction with activated prolactin receptor (aPRLR)

Because the adenovirus technique did not appear to allow long term expression of a transgene, we made transgenic mice using the gene for the activated prolactin receptor on a mammary specific promoter. We were able to obtain expression of the gene in the mammary epithelium but to date (about 1 year after founders were obtained) we have seen no tumorigenesis.

Task 5. Transduction with activated JAK2 and STAT5.

In the laboratory of our original collaborator, Steve Anderson, we used a different member of the prolactin signal transduction pathway, activated Akt (also known as protein kinase B or PKB) to make transgenic mice utilizing the mammary specific promoter, MMTV. These mice have been extensively analyzed and show defects in alveolar development, a reduction in milk production in lactation, and very delayed involution. A few mice have been subjected to multiple pregnancies and two have so far developed tumors. These experiments suggest that the prolactin receptor pathway is involved in tumorigenesis. These results also suggest that, if persistence of a transgene for 12 weeks or longer can be achieved, the interaction of Akt with a known tumorigenic gene will be a worthwhile of the utility of viral transduction for studies of tumorigenesis. However, a retroviral approach would seem more feasible.

Task 6. Evaluation of tumorigenesis

Tumorigenesis has been evaluated in transgenic mice using one of the proposed constructs as described above and a second gene, which is a member of the prolactin signal transduction pathway. In preparation for tests of the utility of adenovirus for studies of tumorigenesis we evaluated the latency of tumor development in mice that overexpress the *neu* oncogene. We have found that mammary tumors begin to appear at six months in this model (figure 5). Unfortunately we were not able to carry out the experiments as planned, because of the limited time over which adenoviral transduction is meaningful.

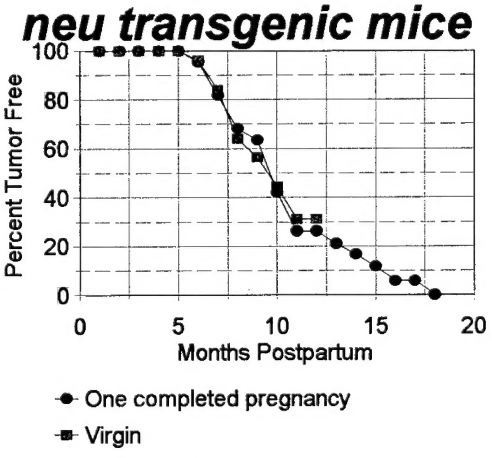

Figure 5. Tumor latency in neu transgenic mice. Mice were either bred at 8 weeks of age and allowed to nurse one litter or they were maintained as virgins. N in each group = 25.

Task 7. Evaluations of alterations of signal transduction pathways.

This task is in progress using the mice overexpressing activated akt under the MMTV promoter. In particular cellular localization of both total and phosphorylated Akt is being visualized. Both forms of the protein can be seen in cells, however, detailed analysis of expression has only begun at this point.

(6) KEY RESEARCH ACCOMPLISHMENTS

Our key accomplishments are:

- Construction of viruses suitable for expression of GFP, Lac Z and the cytoplasmic tail of occludin..
- Putting intraductal injection in the mouse on a firm technical basis, with a technique that can be described and taught to others (see published paper below)
- Achieving reproducible expression of GFP in a high proportion of mammary cells and alveoli under conditions where no inflammation can be detected in the gland 3 - 7 days post-transduction.
- Evaluation of the functional competency of transduced mammary epithelium
- Proof of principle that gene function can be examined in the adenovirus transduced mammary gland.
- We have provided firm evidence that adenovirus is not expressed on a long term basis in the mouse mammary gland and therefore cannot be used directly to examine tumorigenesis induced by oncogenes. It could possible be used in the Cre-lox context to delete certain genes in cells containing floxed genes.
- Use of transgenic technology to show that overexpression of an activated PRL-R in the mammary epithelium does not lead to mammary tumorigenesis
- Use of transgenic technology to show that overexpression of Akt, a member of the prolactin signal transduction pathway, has effects on the mammary gland which include alterations in milk secretion, a delay in mammary involution following lactation and, potentially, enhanced mammary tumorigenesis.

(7) REPORTABLE OUTCOMES

Abstracts:

Injection of adenovirus vectors in the mouse mammary galnd acheives efficient, long term transduction of mammary epithelial cells without inflammation. Neal Beeman and Margaret C. Neville. American Society for Cell Biology, December 1999.

Transduction of Epithelial Cells by intraductal injection of adenovirus vectors into the mouse mammary gland. Margaret C. Neville and Neal Beeman. Era of Hope Meeting, June 2000.

The expression of N-terminally truncated occludin causes programmed cell death in the mouse mammary gland epithelium and in a non-transformed mouse mammary epithelial cell line. Neal Beeman and Margaret C. Neville. American Society of Cell Biology , December, 2001

Papers

Intraductal injection into the mouse mammary gland. 2000. Duy-Ai Nguyen, Neal Beeman, Michael Lewis, Jerome Schaack, Margaret C. Neville. In M.M. Ip and B.B. Asch, *Eds, Research Methods in Mammary Gland Biology and Breast Cancer. Kluwer Academic/Plenum Publishers.pp 259 - 270.** Attached

Mammary gland involution is delayed by activated akt in transgenic mice. 2001. Schwertfeger KL, Richert MM, Anderson SM.. *Mol Endocrinol*, **15**:867-881.

The work in this paper from a collaborator originally on this grant supplanted the transduction of the activated PRLR, but was not directly supported by the money from this grant.

Papers in preparation:

Adenovirus transduction of the mouse mammary epithelium: Strengths and limitations as a means of altering gene expression in the mammary gland. Tanya Reed, Neal Beeman, Emily Freed, Jerome Schaack, Margaret C. Neville. To be submitted to *Breast Cancer Research.*

Expression of a truncated mutant of occludin causes programmed cell death in the mouse mammary epithelium. Neal Beeman, Tanya Reed, Linda Hanson and Margaret C. Neville. To be submitted to *J. Cell Biology.*

Persons receiving salary from this grant:

Linda Hansen, Professional Research Assistant (Mouse breeding and handling)
Neal Beeman, Graduate Student (Intraductal injection, evaluation of tight junction permeability and secretory activity of mammary gland)
Valerie Sawicki, Professional Research Assistant (Mouse breeding and handling)
Tanya Reed, Professional Research Assistant (Intraductal injection, Quantitative analysis of immunofluorescence)
Brigitte Troskie, Ph.D., Postdoctoral Fellow (Immunocytochemistry)
Carol Palmer, Ph.D., Postdoctoral Fellow (Immunocytochemistry, Tissue culture)

Other persons involved in this research who did not receive salary from the grant:

Margaret C. Neville, Ph.D., P.I. (Research Direction, publication)
Steven Anderson, Ph.D., Co-P.I. (Research Direction, transgenic mice)
Jerome Schaack, Ph.D., Co-P.I. (Construction of adenovirus)
Kaylie Schwertfeger, Graduate Student (Evaluation of transgenic mice)
Monica Richert, Ph.D., Post-doctoral fellow (Evaluation of transgenic mice)

(8) CONCLUSIONS

We have made good progress in establishing adenovirus transduction as a method for the short term alteration of gene expression in the mammary epithelium. Thus, detailed conditions for reasonably efficient transduction of the mammary epithelium of the late pregnant mouse have been worked out, transduction with adenovirus and expression of green fluorescent protein, GFP, have been shown not to interfere with function of the mammary cells in which they are expressed. Transgenic technology has been used to evaluate the effects of an activated prolactin receptor, aPRL, and an activated member of the prolactin signal transduction pathway, Akt on the mammary epithelium. The latter has been shown to prolong mammary involution and preliminary results suggest it may enhance tumorigenesis.

Having done this we have carried out a detailed study of transduction efficiency and persistence during the reproductive cycle using an adenovirus with the gene for GFP. The virgin gland transduced efficiently, however, significant inflammation was observed. Reduction in the amount of virus transduced might alleviate the inflammatory response. Transduction was not seen in early pregnant glands, presumably because of the rapid rate of cell division at this time. Transduction efficiency was high when virus was injected during late pregnancy and persisted throughout lactation. Inflammation, as detected by increased tight junction permeability and appearance of lymphocytes on histological sections, was not a significant problem within 7 days after transduction, but became a problem at 10 days after transduction, presumably due to an immune response to viral products. Finally, transduction of an adenovirus containing a truncated occludin molecule expected to cause disruption of tight junctions did so in a programmed manner and eventually brought about programmed cell death of the transduced cells. Because this is precisely the response obtained in transduced cells in tissue culture we consider this final experiment to constitute a proof of principle that adenovirus transduction can be used to express foreign genes in the mammary epithelium with functional consequences. The technique is appropriate for short term experiments, up to 7 days, but cannot be used for long term expression of foreign proteins because of inflammatory reactions.

(9) REFERENCE

1. Bamforth SD, Kniesel U, Wolburg H, Engelhardt B, Risau W J A dominant mutant of occludin disrupts tight junction structure and function. 1999 Jun;112 (Pt 12):1879-88.

(10) APPENDICES

Intraductal injection into the mouse mammary gland. 2000. Duy-Ai Nguyen, Neal Beeman, Michael Lewis, Jerome Schaack, Margaret C. Neville. In M.M. Ip and B.B. Asch, *Eds, Research Methods in Mammary Gland Biology and Breast Cancer. Kluwer Academic/Plenum Publishers.pp 259 - 270.*

Chapter 23

Intraductal Injection into the Mouse Mammary Gland

Duy-Ai Nguyen, Neal Beeman, Michael Lewis, Jerome Schaack, and Margaret C. Neville

Abstract. The mammary epithelium is continuous with the skin through a teat canal leading to a single primary duct in the mouse. Using fire-polished micropipettes 60 to 75 μm in diameter, it is possible to inject any desired substance directly through the teat into the lumen of the mammary gland. If the primary duct of the gland is exposed surgically, hypodermic needles can also be used for injection. Both techniques can be used to investigate the state of tight junctions in the mammary gland by examining transepithelial movement of radioactive sugars or fluorescent-labeled proteins. The intraductal or up-the-teat injection of adenoviral and plasmid vectors provides a convenient means of altering gene expression in the luminal epithelium. Finally, injected fluorescent probes as well as adenovirus-transduced green fluorescent protein can be directly visualized in the mammary gland in the living mouse by using confocal microscopy.

Abbreviations. green fluorescent protein (GFP); bovine serum albumin (BSA); fluorescein (FITC); a blue nuclear stain (DAPI).

The mammary epithelium is accessible from the exterior of the animal through the teat canal. This unusual characteristic is technically advantageous and was exploited nearly 30 years ago by James Linzell and his colleagues for assessing the transepithelial permeability of the mammary gland to a variety of substances in the pregnant and lactating goat (1–3). Falconer injected ^{125}I-prolactin intraductally in rabbits to assay its distribution. More recently, intraductal injection of the mammary gland has been used in the rat for transduction of the epithelium with retroviruses (Chapter 22 this volume) and adenovirus (4, 5). Intraductal injection of antitumor agents has been proposed for breast cancer therapy in women, but reports of its efficacy do not seem to be available in the literature. This powerful technique has not been much exploited in the mouse, because of the small size of the teats and major mammary duct. This difficulty can be overcome with the use of appropriately sized micropipettes inserted directly into the teat canal through the nipple (up-the-teat injection) or with the use of large-gauge needles inserted into the primary duct of the surgically exposed mammary gland. In this laboratory the technique has been used to assess the status of the tight junctions between

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Methods in Mammary Gland Biology and Breast Cancer Research, edited by Ip and Asch. Kluwer Academic/Plenum Publishers, New York, 2000.

epithelial cells by measuring the permeability of the mammary epithelium to radioactive sucrose and to fluorescent-labeled proteins. It has also been used for adenoviral transduction and transfection with plasmids and for visualization of the mammary gland *in vivo.*

In this chapter we describe fabrication of the micropipettes used for up-the-teat injection. For the injection we utilize a dissection microscope and a drawn micropipette mounted on a micromanipulator to enter the teat canal. An alternative technique uses surgical exposure of the third or fourth mammary gland and injection with a Hamilton syringe directly into the primary duct. We illustrate the use of up-the-teat injection to measure transepithelial permeability, using [^{14}C]-sucrose and fluorescently labeled proteins. Transduction of the luminal epithelium with an adenoviral vector and visualization of the mammary lumen with the confocal microscope in the living mouse are also described briefly.

MATERIALS

General

- Anesthetic: pentobarbital, 60 μg/gm body weight with 10 to 20 μg/gm as follow-up dose or as specified by your Institutional Animal Care and Use Committee
- Dissecting microscope
- Fiber-optic light source

Fabrication of Micropipettes for Up-the-Teat Injection

- Bunsen burner with flame spreader
- Fine forceps (old ones that can be put in the flame)
- 10-μl or 25-μl glass micropipettes (Drummund precision disposable micropipettes on 10 or 25 μl or Wiretrol calibrated micropipettes with stainless steel plunger, up to 100 μl)
- Compound microscope with calibrated ocular reticle and adjustable stage
- Micromanipulator
- Platinum wire electrode
- Variable power source

Up-the-Teat Injection

- Prepared micropipette with 60 to 75 μM tip (see above)
- Drummond digital pipette (10 or 100 μl)
- Micromanipulator
- Blunt-tipped forceps
- High-intensity fiber-optic light
- Electric light to keep mouse warm

Intraductal Injection

SURGICAL

- Dissecting microscope and fiber-optic light source
- Cork board (4 inch × 6 inch)
- Straight pins

- Surgical tape
- 70% ethanol
- Opening scissors
- Forceps—mouse tooth and smooth
- Cotton-tipped wooden applicators
- Wound clips or suturing supplies
- Recovery chamber, warm
- Black "Sharpie" or magic marker (optional)

EXPERIMENTAL

- Injectable substance (viruses, plasmids, tracer dyes, etc.)
- 30G1/2 needle or higher (Becton Dickinson & Co., Franklin Lakes, NJ 07417)
- Hamilton syringe with hub for removable needles (50 µl) or similar
- Tracer dye (optional), e.g., 0.1–0.5% trypan blue or Evan's blue in 1× PBS or 0.9% saline

Measurement of Transepithelial Permeability

- ^{14}C-sucrose (2×10^6 cpm/8 µl)
- Drummond digital microdispenser set to 8.8 µl
- Injection apparatus as above

Observation of the Mammary Alveolus in a Living Mouse

- Inverted confocal microscope with removable stage diaphragm
- Heating pad
- Large coverslips
- Duct or surgical tape
- Anesthetic (pentobarbitol, 5 mg/ml; inject 0.01 ml/g body weight)
- Fine scissors
- Forceps
- Blunt probe
- Gauze pads
- Ringer's solution

METHODS

Fabrication of Micropipettes for Up-the-Teat Injection

The goal is to pull a calibrated micropipette (Drummund Precision Disposable micropipettes, 10 or 25 µl) so that it has a strong, tapered, and polished tip 60 to 75 µm in diameter. The trick in achieving proper tip size and conformation is to pull each pipette in two stages. The first pull is slow and reduces the diameter by a factor of 2; the second pull is rapid and results in a gradual taper to a diameter less than 75 µm. The pipette is then broken under the microscope and fire-polished in a microforge.

DETAILS. Attach a flame spreader to a large Bunsen burner or fashion a flame spreader from heavy foil. Adjust the flame to 1 inch. Insert one tine of the forceps into the lumen of the dispensing end of the pipette and grasp the glass gently. Hold the plunger end with the fingertips. Hold the dispensing end of the pipette at the top of the flame with the pipette level and

perpendicular to the band of flame. Maintain light tension only so that the wall thickness is unchanged and the glass melts to form an hourglass shape (Figure 23-1A, pipette 1). When the dispensing end begins to move, remove the pipette from the flame as rapidly as possible while keeping the glass tube as straight as possible. Dip the dispensing end in water to cool. Water will be drawn up into the pipette. The external diameter at the waist of the pulled section is about one-half the original diameter of the tube (Figure 23-1A, pipette 1).

For the second pull hold the pipette as before, but exert tension horizontally throughout the pull. Expose the pipette to the flame as before, with the band of flame at the waist of the hourglass. The water in the lumen may boil but this will not affect the correct pull. As the pipette becomes plastic, both hands begin to move apart rapidly. At this point remove the pipette immediately from the flame while maintaining horizontal tension and dip the dispensing end in water to cool. Water should be drawn into the pipette past the waist of the elongated hourglass. The waist of the hourglass should be 75 μm or less in external diameter at its narrowest point; the wall of the pipette at this point is very thin, and often flexible (Figure 23-1A, pipette 2). If water is not drawn into the pipette, it has been sealed. The end of the pipette is often pulled off accidentally during this step. These pipettes and the sealed ones should be examined under the microscope to determine whether the lumen is patent. If not, discard them.

The pulled tubes are broken with fine forceps under a dissecting microscope to obtain a tip of proper diamenter. The ocular reticle of a *compound* microscope is used to locate the region of the pipette with an external diameter between 60 and 75 μm. The shape of this target region is carefully noted and the pipette is placed flat on the stage of a *dissecting* microscope. The pipette is grasped just below the target diameter with fine forceps held flat against the stage. The forceps are rotated gently to force the dispensing end upwards and snap it off. If the forceps are maintained at the level of the stage and perpendicular to the pipette, the dispensing end will snap off at the desired diameter and a reasonably smooth break will be obtained (Figure 23-1A, pipette 3). Once a pipette of ideal tip diameter is obtained it can be used as a guide in breaking other tips correctly under the dissecting scope.

The broken pipettes are polished on the stage of the *compound* microscope with a platinum wire electrode (or microforge) held in a micromanipulator (Figure 23-1B). The broken pipette is placed flat on the microscope stage so that the tip is just visible with a 10× objective. Using the micromanipulator, advance the tip of the microforge into the other side of the field of view (M, Figure 23-1B). The variable power source is used to adjust the power gradually upward until the wire just begins to glow in the microscope field. The wire loop will extend forward slightly as it is heated. When the pipette tip and the tip of the microforge are brought within 3 to 4 μm of each other, a smoothly broken tip will be polished rapidly and should be removed as soon as the tip appears smooth. More jaggedly broken tips can often be polished smoothly if they are positioned carefully near the electrode so that jagged projections are closer to the heat source. It is often necessary to reposition such a tip several times during polishing. Polished tips do not have to be perfectly regular. Two finished tips are shown in Figure 23-1C.

Before attempting an injection, always test the pipette to make certain that fluid can be drawn up and expelled freely. A pipette can be used until it is broken or clogged. For cleaning, the pipette can be attached to a vacuum apparatus (Figure 23-1D) and large volumes of fluiddrawn rapidly through it. To clean pipettes, 70% ethanol followed by sterile water is recommended.

Up-the-Teat Injection

ANIMAL PREPARATION. For injection into the lumen of the mouse mammary gland through the teat canal (up-the-teat injection), the mouse is positioned on a platform beneath a

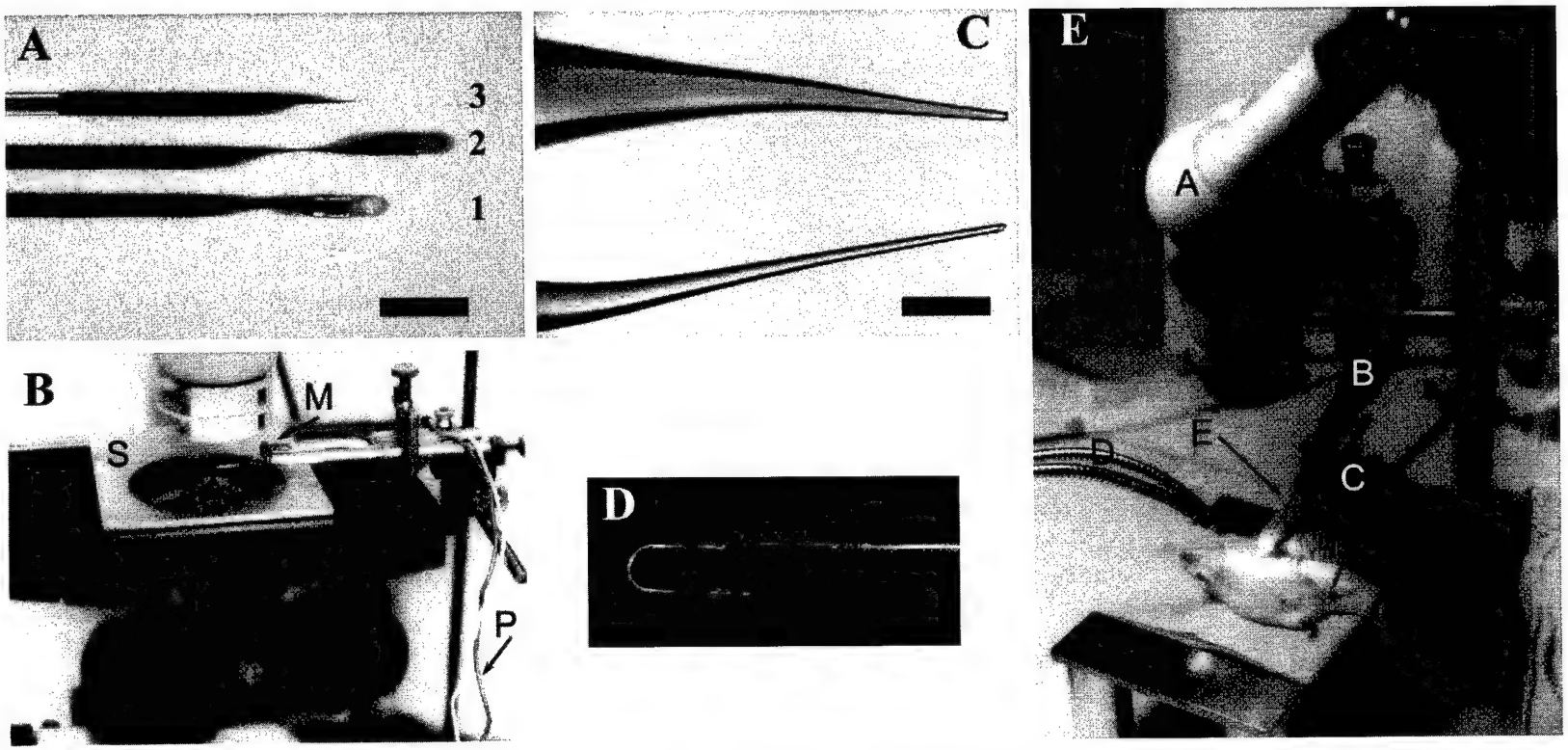

Figure 23-1. Fabricating and using micropipettes for up-the-teat injection. (A) Stages in pulling micropipettes. After a slow pull the heated portion of the pipette has an hourglass shape and the wall has not appreciably thinned (bottom pipette). When the pipette is pulled a second time, the waist of the indentation narrows to less than 75 µm (middle pipette) or breaks entirely (top pipette). (Pipettes are filled with dye solution to facilitate photography.) (B) Microforge (M) positioned over movable stage (S) of a compound microscope with wires to a variable power source (P). The platinum heating wire can be seen above the objective in the opening on the movable stage. For fire-polishing, a freshly broken micropipette is placed on the left-hand side of the stage with the tip about 4 µm from the platinum wire. (C) Tips of two fire-polished pipettes. Bar is 100 mm. (D) A Pasteur pipette attached to a piece of flexible tubing and a micropipette for cleaning. (The micropipette was taped down for the purposes of the photograph.) (E) Setup for teat canal injection using a Drummond microdispenser (B) mounted on a micromanipulator (C) beneath a dissecting microscope (A). The micropipette (E) is inserted into the teat canal by manipulating the tissue surrounding the nipple with a pair of fine forceps.

dissecting microscope (Figure 23-1E, A). A micropipette (E) inserted into a Drummond digital microdispenser (B) held in a micromanipulator (C) is positioned above the second, third, or fourth nipple that has been prepared as will be described. A fiber-optic light (D) is convenient for visualizing the field. The tip of the micropipette is inserted into the lumen of the canal, and 8 to 25 µl of solution is injected. For larger volumes a Wiretrol micropipette (5 to 100 µl) can be held in the micromanipulator with a length of narrow-bore rubber tubing to fit the pipette to the micromanipulator, and the solution can be dispensed with the accompanying stainless steel plunger.

The first step is to prepare the nipple for injection. Removing the hair around the nipple to obtain a clear unobstructed work area can facilitate the injection procedure. For lactating mice, the opening for the teat canal is readily apparent at the center of the teat, and the capillary tip will readily slip in when pushed against the nipple at this location. Staining the teat with trypan blue will make the opening more visible in the beginning. In nonlactating mice, the opening to the teat canal is often covered with a layer of dead skin that must be removed by repeated gentle grabbing and pulling with a pair of microscissors. Although the layer of dead skin can also be removed by cutting off a thin layer of skin at the tip of the nipple, excessive cutting can deform the nipple and makes the task of locating the opening more difficult.

INJECTION. To insert the tip of the capillary tube into the teat canal, the tube is first positioned immediately adjacent to the base of the nipple with the micromanipulator. The center of the nipple is then positioned under the tip of the capillary by manipulating the skin around the nipple with a pair of blunt forceps. Direct manipulation of the nipple is possible, but can result in damage. Next, the center of the nipple is pushed upward against the tip of the capillary by using the natural elasticity of the nipple or a light upward motion of the forceps. If correctly performed the tip of the capillary should enter the teat canal easily. If it does not, then the tip may not be positioned properly at the opening of the teat canal or the opening is still covered by skin. A delicate touch is critical to these procedures.

Finally, the material in the capillary tube is injected into the lumen of the mammary gland. Since the tip of capillary was initially positioned at the level of the base of the nipple, the nipple will be under compression and exert an upward force against the capillary tip. This upward push helps to prevent the capillary tip from slipping out of the teat canal during slight movements of the mouse, such as breathing. The nipple and the inserted capillary tip should be observed carefully throughout the injection because even slight movement can dislodge the tip from the teat canal.

Intraductal Injection

For younger or nulliparous virgin mice, injection through the teat canal is difficult. In this case the gland can be exposed surgically and the primary duct just below the teat injected with a Hamilton syringe.

ANIMAL PREPARATION. The mouse is anesthetized and affixed on its back to a cork board with surgical tape or straight pins through the paw webbing. The ventral fur is wetted with 70% ethanol to reduce fur entry into the wound. If the animal has a light coat color (e.g., BALb/c, CD1) the nipple region may be "painted" with a black magic marker to provide better visualization of the nipple and primary duct.

A 1.5–2 cm mid-ventral incision is made beginning just above the pubic area. Two additional incisions are made from the base of the incision at an angle toward the midpoint of the hind legs. The angled cuts should be well behind the nipple for the number 4 mammary gland.

The triangular patch of skin and the attached number 4 mammary gland are peeled back gently with mouse-tooth forceps and a cotton-tipped wooden applicator and pinned to the cork board such that the skin is tight and the gland is roughly flat.

INJECTION. Locate the nipple under the dissecting microscope: it will appear as a grayish circular spot against a lighter background of the skin. The nipple should be near the corner created by the two incisions but not so close that the pin will interfere with the injection.

Next, locate the primary duct of the mammary gland. The primary duct extends from the nipple back toward the no. 4 fat pad. If necessary, use the smooth forceps to wiggle the fat pad gently. The location of the primary duct should become apparent since it is attached to the nipple and will move with the fat pad.

Load the syringe with approximately 25–30 µl of the injectable substance; there will be some dead airspace in the syringe. Using an oblique angle of attack in the same orientation as the length of the primary duct, carefully insert the tip of the needle a few millimeters into the lumen of the primary duct near the nipple. The skin may need to be held with forceps to facilitate needle penetration. Take care not to penetrate the back wall of the duct. Slowly inject 10–20 µl of the substance into the gland. If done correctly, the entire mammary ductal tree will fill with the injected fluid. The exact volume tolerated by the gland before the terminal end buds or duct termini burst varies with the age of the animal and the extent of gland development and should be determined empirically by using a tracer dye on a few test animals.

After injection, wait about 5 s for the back pressure to reduce; then use smooth forceps to gently squeeze the fat pad around the needle and slowly pull the needle out of the duct. Pinch the duct with the forceps as the needle passes the tip and hold to prevent leakage of the injected substance. Close the animal with wound clips or sutures and allow recovery in a warm chamber or fresh cage. Repeat for the contralateral gland.

Injection of Adenovirus

ADENOVIRUS PREPARATION. Adenovirus preparation has been described in detail in Li *et al.* (Chapter 21 this volume).

For these experiments adenovirus containing green fluorescent protein (GFP) or *lacZ* under control of the cytomegalovirus major immediate early promoter (CMV promoter) was used. Adenoviral E1A and E1B genes within the left end of the adenovirus chromosome were deleted so that the virus was replication defective, although replication was not completely eliminated (6).

TRANSDUCTION OF THE MAMMARY EPITHELIUM *IN VIVO*. We have found that adenovirus transduction is more efficient when the virus is injected into the gland of the pregnant animal. Presumably the presence of large amounts of milk in the lumen interferes with infectivity in the lactating animal. For observations during lactation we carry out the injections on day 19 of pregnancy, injecting $5–8 \times 10^7$ pfu (plaque-forming units) of virus particles diluted with Ringer's solution containing 10% calf serum. In general, we inject a volume of 50–200 µl. Although we often assay the mouse at 3 days of lactation, GFP expression has been found to persist through late lactation.

Observation of the Mammary Alveolus in a Living Mouse

Fluorescent probes can be injected up the teat or intraductally to visualize the mammary lumen in the live mouse. Adenovirally encoded fusion proteins containing GFP can also be

visualized *in vivo*. The fourth mammary gland is the easiest to visualize with the anesthetized mouse placed on the stage of an inverted microscope equipped for laser or digital confocal fluorescence microscopy.

PREPARATION OF THE ANIMAL. The microscope stage is warmed with a stage heater (ideal) or heating lamp. If a heat lamp is used it must be turned off while taking micrographs. The stage insert is removed from the microscope and placed with a 50-ml centrifuge tube of Ringer's solution on a heating pad to warm. A coverslip is taped over the aperture in the insert.

The mouse, anesthetized as described, is placed on its back on a warm heating pad. A 2-cm mid-ventral incision is made beginning midway between the fourth and fifth teats. A second incision is made from the base of the mid-ventral incision at an angle toward the mid-point of the hind legs, passing over the junction of the fourth and fifth mammary glands. A third incision is made at the anterior end of the midline incision angled laterally between the third and fourth mammary glands, leaving a wide margin of skin around the fourth mammary gland. A blunt probe or cotton-tipped applicator is worked along the length of the three incisions and is used to lift the ventral two-thirds of the fourth mammary gland entirely free of the body wall while preserving mammary circulation.

The mouse is now placed prone on the warmed stage insert and the deflected flap of skin is arranged on the secured coverslip over the aperture. The mammary gland should be visible through the aperture. In general, most fluorescent probes can also be seen with the naked eye, an aid in positioning the animal. Tape the flap of skin lightly to the insert, taking care not to compress the gland. A flaccid fold of skin between the mouse and the taped area helps to reduce the transmission of breathing movements to the visualized tissue. Place a gauze pad dampened with warm Ringer's solution beneath the mouse, making sure that the gauze contacts all exposed tissues. The edge of the gauze pad should extend over the edge of the coverslip but not across the aperture. A film of saline will spread between the coverslip and the visualized tissue and prevent drying.

VISUALIZATION OF THE MAMMARY GLAND. The stage insert bearing the mouse is placed on the stage of the inverted microscope and the mammary gland is visualized through the objective. To prolong visualization of a living gland, keep the gauze pad well moistened with Ringer's solution and maintain body temperature of the mouse. A microscope with a heated stage is ideal for this experiment. Alternatively, a heat lamp may be placed over the microscope stage to keep it warm but must be be switched off while taking micrographs. The stage should remain warm to the touch but not allowed to become hot. A dark redness of the ears, feet, and tail indicates overheating. Etiolation or whitening of these areas indicates underheating. If the anesthetic begins to wear off, small doses may be injected intraperitoneally through the ventral body wall by simply rolling the mouse toward the tape.

Throughout the experiment red blood cells should be seen coursing through the vasculature of the gland, indicating adequate circulation. Although it is occasionally possible to obtain usable images with a 100× oil emersion objective, because most structures lie more deeply in the tissue a long working distance 60× objective is generally more satisfactory. In addition, best results are obtained in a mouse that has been lactating for 10 days or more because the thickness of the adipose layer that generally surrounds the parenchyma is reduced at this time. More detailed discussion of the use of digital or laser confocal microscopy is beyond the scope of this chapter.

RESULTS AND DISCUSSION

In this section we give examples of the application of up-the-teat injection to studies of mammary function.

Measurement of Transepithelial Permeability

The teat canal injection procedure can be applied to the assessment of permeability of tight junctions between the mammary epithelial cells *in vivo*. Tracer injected into the lumen of the mammary gland will remain there if the tight junctions are closed. However, if they are open the injected tracer will leak out of the lumen and into the interstitial space. Since small molecules such as sucrose can readily transfer across the endothelium of the capillaries and enter the bloodstream, the peak level in the blood mirrors the leakage rate of such molecules. In our study, we injected the tracer [^{14}C]-sucrose into the lumen of the mammary glands during pregnancy and lactation. During pregnancy, [^{14}C]-sucrose appeared in the bloodstream almost immediately after injection, as the tracer leaked across the permeable tight junctions (Figure 23-2). The level in the bloodstream then fell with a half-time of about 20 min as the nonmetabolizable sugar was cleared by the kidneys. During lactation, injected [^{14}C]-sucrose was not detectable in blood samples, because it was confined to the lumen by the impermeable tight junctions of lactation.

The movement of large tracer molecules, such as proteins that might not cross the capillary endothelium, can be followed by using fluorescently labeled proteins and fluorescent microscopy. Figures 23-3A, B show the results of an experiment in which FITC–BSA was injected into the lumen of the mammary glands of deeply anesthetized mice during pregnancy (A) and lactation (B). The probe was followed immediately by injection of 4%

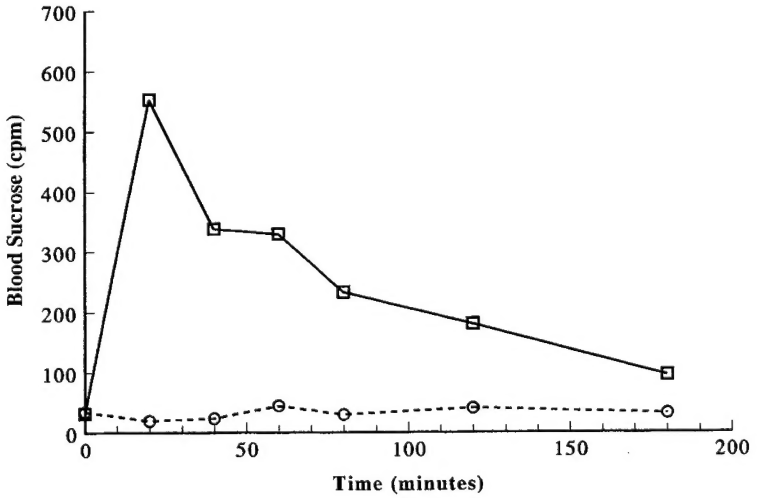

Figure 23-2. Appearance of [^{14}C]-sucrose in the bloodstream after microinjection of 10^6 cpm into the lumen of the mammary gland in pregnant and lactating mice. After injection, the [^{14}C]sucrose level in the bloodstream rapidly increased then declined in the pregnant mouse as the isotope was cleared by the kidney (open squares). The level of [^{14}C]-sucrose remained at background in the lactating mouse (open circles).

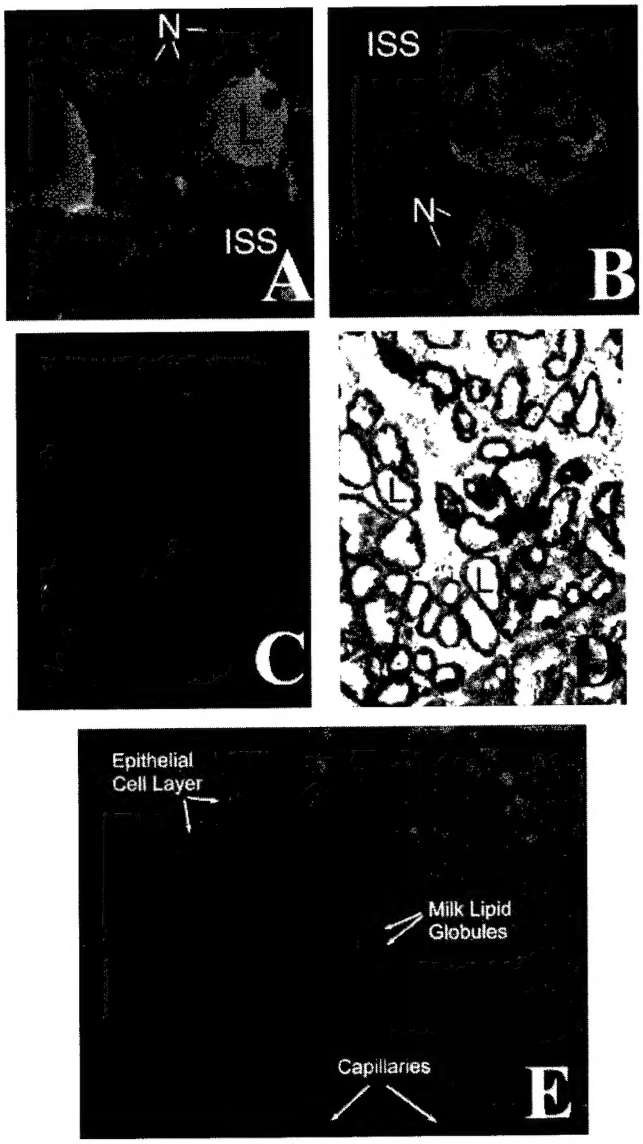

Figure 23-3. Views of injected mammary gland. (A) Distribution of FITC–albumin in the mammary glands of a pregnant mouse. Several alveoli with green fluorescence from the FITC–albumin in the lumina are shown. Nuclei (N) are stained with DAPI (blue). Note the leakage of the FITC–albumin from the lumen into the basolateral and interstitial space (ISS) of the mammary alveoli. (B) Distribution of FITC–albumin in sections of mammary glands of a lactating mouse. The FITC–albumin is confined to the lumen. 400×. (C) Adenovirus transduction of mammary epithelium. Low-power view (40×) on the FITC channel of GFP transduction of fourth mammary gland visualized in an anesthetized mouse prepared as described in text. (D) Section of mammary gland transduced with virus containing gene for *LacZ*. Section stained with Bluo-gal (Sigma) as described by Sanes *et al.* (7). (E) Visualization of the mammary lumen and nuclei of mammary alveoli in the live mouse. The fourth mammary gland of a 10-day lactating mouse was injected with Cy3-labeled IgG and the vital dye DAPI. The nuclei of the alveolar cells are shown in blue. Dark lines crossing the alveoli are capillaries. Blood cells can be seen to move in these vessels under transmitted light (not shown). (For a color representation of this, see figure facing page 268.)

paraformaldehyde in PBS up-the-teat to fix the tissue. After dissection the gland was embedded in polymethacrylate embedding medium (JB4), sectioned, and viewed with the digital confocal microscope. Subsequent fluorescent microscopy showed that during lactation, the FITC–BSA was present exclusively within the lumen (Figure 23-3B), while during pregnancy the FITC–BSA was found throughout interstitial space of the mammary gland as well as in the lumen (Figure 23-3A). Although data obtained using protein probes are less quantitative than those using radiolabeled tracer, such probes do allow the leakage path of the tracer to be followed morphologically. In this case the tracer allowed us to ascertain that the probe leaked around the cells rather than being transcytosed, since the injected FITC–BSA was seen in the paracellular space but was not detectable within the mammary epithelial cells.

Adenoviral Transduction of Luminal Epithelium

Figure 23-3C shows a low-power view of a mammary gland from a 3-day lactating mouse transduced with adenovirus containing the GFP gene on day 19 of pregnancy. At this magnification, which does not distinguish individual cells, a large proportion of the gland appears to be transduced. In order to better estimate the proportion of cells transduced, a mouse was similarly injected with a virus containing the *lacZ* gene. The gland was dissected, reacted with the *lacZ* chromagen Bluo-gal (Sigma) to obtain the characteristic blue color and processed for frozen-section microscopy. A representative section is shown in Figure 23-3D. Many cells in the section stain a very dark blue, indicating a high degree of expression. In some alveoli all the cells appear to be stained. These alveoli are expanded and contain evidence of milk in the lumen, suggesting that neither the adenovirus transduction nor the presence of the foreign gene, *lacZ*, interferes with the normal function of the gland. However, the proportion of cells transduced is small. This degree of transduction needs to be kept in mind when experiments are planned, particularly if morphological assays are not to be used. If the criteria for the effect of a particular gene substitution are morphological, then the presence of adjacent transduced and untransduced cells and alveoli provides a control for the effect of the transgene.

Visualization of the Mammary Lumen and Epithelium *In Vivo*

We have found that the lumen of the mammary gland can be visualized with injection up the teat of fluorescent dyes. Figure 23-3E shows a digitally deconvolved view of the mammary gland of a living mouse that had received an injection of 150 µl of Cy3-labeled IgG (0.375 mg/ml) with 40 µg/ml DAPI, to visualize the nuclei. The milk fat globules are clearly visible in the lumen of many of the alveoli, and the DAPI has indeed rendered the nuclei visible. We hope to use this technique, perhaps in conjunction with lipophilic dyes, to visualize milk fat globule secretion. The possibility that calcium binding dyes can be used to quantitate the level of intracellular calcium in various stages of lactation is intriguing. We have just begun to explore this area and are still working out the possibilities and limitations of the technique.

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